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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: IMPROVING POLYNUCLEOTIDE LIGATION REACTIONS

(57) Abstract: The method of the invention improves the specificity of a ligation reaction carried out between a first double-stranded polynucleotide having a single-stranded portion and a second polynucleotide having a complementary single-stranded portion, the second polynucleotide being present in a sample comprising a mixture of different polynucleotides. The method comprises contacting the sample, under hybridising conditions, with the first polynucleotide and one or more third polynucleotide(s) wherein the third polynucleotide(s) comprises a single-stranded portion that differs from the single-stranded portion of the first polynucleotide by at least one base substitution.

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Improving Polynucleotide Ligation Reactions

Field of Invention

The present invention relates to methods for improving polynucleotide ligation reactions.

Background to Invention

Berg and Boyer created the first recombinant DNA molecule in 1972. This simple concept of recombination - the splicing together of two pieces of DNA and fusing them by ligation, is the basis for the entire field of molecular biology. Molecular biology has become ubiquitous to the point where it is central to the majority of all biological research. The ligation reaction is performed thousands of times a day in research and diagnostic laboratories worldwide. Given the boundless opportunity presented by genetic engineering, the ligation reaction is likely to remain a central technique for many years to come.

The ligation reaction itself is chemically simple, comprising the linking of two nucleotides by the creation of a phosodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another, by a ligase enzyme. There are two types of ligation, known as "sticky end" and "blunt end", depending on the presence or lack (respectively) of complementary single stranded regions on the two polynucleotides to be joined, in proximity to the ligation location. "Sticky-end" ligations involve the hybridisation of complementary single stranded sequences between the two polynucleotides to be joined, prior to the ligation event itself. Sequences that have similar but not 100% complementary single stranded sequences will still be ligated, known as a mismatch ligation. These result in the ligation of an incorrect sequence and decrease the efficiency and fidelity of the overall ligation reaction.

Since ligation is such an important reaction, ligases are available on the market that are improved, modified and optimised to give maximum efficiency. These enzymes are expensive and it is therefore desirable to use as a small amount as is possible without reducing the efficiency of the reaction and whilst avoiding mismatch ligation. Mismatch ligations are problematic as they are

deleterious to the fidelity of the ligation process. It is therefore desirable to minimise mismatch ligations.

Current methods of increasing ligation specificity include decreasing the amount of ligase and increasing the salt in the reaction mix to slow down the reaction. Since match ligations are much faster than mismatch ligations, the increased specificity observed using this technique is a result of the slower reaction speed and whilst this increases the match: mismatch ratio, it results in a low yield and does not prevent mismatch ligations.

There is therefore a need for improvements in ligation reactions.

Summary of Invention

The present invention is based on the realisation that specificity in "sticky-end" ligations can be increased by including short adapters that reduce the occurrence of mismatch ligation.

According to a first aspect of the invention, a method for improving the specificity of a ligation reaction carried out between a first double stranded polynucleotide having a single stranded portion and a second polynucleotide having a complementary single stranded portion, said second polynucleotide being present in a sample comprising a mixture of different polynucleotides, comprises:

contacting the sample, under hybridising conditions, with the first polynucleotide and one or more third polynucleotide(s), wherein the third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base substitution, and carrying out a ligation reaction.

The present invention improves the yield of match ligations by reducing mismatch ligations through the use of blocking polynucleotides which hybridise to incorrect single stranded overhangs on the second polynucleotides.

Description of the Drawings

The present invention is illustrated by reference to the accompanying drawing, where:

Figure 1 is a graphic illustration of match:mismatch ratio as a function of time, wherein Figure 1a illustrates the ratio in the absence of blocking adapters, and Figure 1b illustrates the ratio in the presence of blocking adapters.

Detailed Description of the Invention

The present invention is used to increase specificity of polynucleotide ligations. The term "polynucleotide" is used herein to refer to biological molecules made up of a plurality of nucleotides. Preferred polynucleotides include DNA, RNA and synthetic analogues thereof, including PNA.

The term "hybridising conditions" is used herein to refer to conditions that allow complementary base pairing to occur between two polynucleotides, such that two complementary single stranded polynucleotides will hybridise to form a duplex. Such conditions are well known in the art. An Example of such conditions is incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. Alternative conditions will be apparent to the skilled person and are applicable to the present invention.

In any ligation reaction, two polynucleotide molecules are joined. The term "first polynucleotide" is used herein to refer to one of the two intended targets of ligation. The term "second polynucleotide" is used herein to refer to the other of the two intended targets of ligation.

A non-limiting example of the terms "first polynucleotide" and "second polynucleotide" comprises the "first polynucleotide" being a DNA vector into which an insert, the "second polynucleotide", is to be ligated to form a recombinant construct.

In any ligation reaction, there may be polynucleotides present which are neither "first polynucleotides" or "second polynucleotides", in the sense that they are not intended to be part of the ligation reaction. These polynucleotides interfere with the ligation between the first and second polynucleotides, which results in mismatch ligations.

As used herein, the term "third polynucleotide" is used to describe polynucleotides which are added to the ligation reaction mixture to hybridise to any polynucleotide which is not a first or second polynucleotide, preventing the unwanted polynucleotides from reacting with the other components of the reaction mix. The third polynucleotides are not totally complementary to the first or second polynucleotides.

The method increases specificity in polynucleotide ligations through the addition of one or more third polynucleotide(s) into a reaction mix. This reaction mix comprises a first polynucleotide and a second polynucleotide, which contain complementary single stranded portions. The second polynucleotide is present in a sample comprising a mixture of different polynucleotides, and is the intended target for binding to the first polynucleotide. The third polynucleotide(s) comprises at least a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base. The number of differences between the first and third polynucleotides may depend on the size of the single stranded portions involved. For example, if the single stranded portion is only 3 bases in length, a single difference may be suitable, but if the single stranded portion is 6 bases in length, multiple differences may be preferred. The differences may be substitution(s), deletion(s) or addition(s).

The third polynucleotide may be added to the sample containing the second polynucleotide simultaneously with or sequentially before or after the first polynucleotide. The third polynucleotide is preferably added to the sample containing the second polynucleotide, along with the first polynucleotide.

Preferably, the third polynucleotide is present in excess with respect to the first and second polynucleotides, to ensure that all other polynucleotides in the sample are hybridised by the third polynucleotide.

It is intended that the first and second polynucleotides hybridise and are ligated together, to the exclusion of other polynucleotides in the sample. The third polynucleotide(s) hybridise to the other polynucleotides in the sample which would otherwise compete for binding to the first polynucleotides, effectively preventing them from hybridising to the first polynucleotides and increasing the

number of correct binding events between the first and second polynucleotides. This increases the specificity of the overall ligation reaction.

Preferably the mixture of third polynucleotides comprises double stranded polynucleotides with a single stranded portion, such that the single stranded portion hybridises its complementary region on incorrect first and second target polymers.

Preferably, the single stranded portion of each of the first, second and third polynucleotides is from 3 to 6 bases in length. Most preferably, the single stranded portion is 4 bases in length.

Figure 1 is a graphical representation of the match:mismatch ratio as a function of time. This ratio becomes lower as the reaction progresses, since the match reaction rapidly reaches plateau and is caught up by the slower mismatch reaction. Traditional methods of increasing specificity merely slow the reaction (using less ligase or increasing salt concentration) and shift the reaction to the left of the graph, where the match:mismatch ratio is favourable but yield is decreased. The present invention ensures that the mismatch ligations do not increase. The match reaction can proceed to full term without the mismatch reaction ever catching up. This provides an optimised match:mismatch ratio.

The invention will now be illustrated with reference to the following, non-limiting, example.

Example

A test system was set up to measure the effect on ligation specificity of adding blocking adapters to a ligation reaction. The goal of this test system was to measure ligation specificity, i.e. the percentage of correctly ligated molecules relative to the total number of molecules ligated. The test system used makes use of the fact that a ligation product containing a single base mismatch in the ligation overhang region differs from correctly ligated ligation product by only one base. The protocol used was developed to measure ligation specificity (match ligation as percentage of total ligation) using the Homogeneous Mass Extend method (Sequenom) and the MassARRAY stystem (Sequenom). The protocol was used to measure the specificity of ligation for the 3'-most base (upper strand) of the ligation overhang region.

The polynucleotides used in this Example are shown in Table 1.

Table I: Sequences of adapters used

DPA 12

5′-
TGTGTCCGCGTGGCTCTTCTATTCTTGGCTTTTCGTCGCTTGGCTTTTCGTCGCTTGGTCATTCGTCGCTTGG
TCATTCGTCGCTTGGCTTTTCGTCGCTTGGTCATTCGTCG -3′ (SEQ ID NO: 1)
3′-
ACACAGGCGCACCGAGAAGATAAGAACCGAAAAGCAGCGAACCGAAAAGCAGCGAACCAGTAAGCAGCGAACC
AGTAAGCAGCGAACCGAAAAGCAGCGAACCAGTAAGCAGCTAA -5′ (SEQ ID NO: 2)

DPA 14

5′-
TGTGTCCGCGTGGCTCTTCTATTCTTGGCTTTTCGTCGCTTGGCTTTTCGTCGCTTGGTCATTCGTCGCTTGG
TCATTCGTCGCTTGGTCATTCGTCGCTTGGCTTTTCGTCG -3′ (SEQ ID NO: 3)
3′-
ACACAGGCGCACCGAGAAGATAAGAACCGAAAAGCAGCGAACCGAAAAGCAGCGAACCAGTAAGCAGCGAACC
AGTAAGCAGCGAACCAGTAAGCAGCGAACCGAAAAGCAGCTAA -5′ (SEQ ID NO: 4)

DPA 24

5′-
TGTGTCCGCGTGGCTCTTCTATTCTTGGCTTTTCGTCGCTTGGTCATTCGTCGCTTGGTCATTCGTCGCTTGG
CTTTTCGTCGCTTGGCTTTTCGTCGCTTGGCTTTTCGTCG -3′ (SEQ ID NO: 5)
3′-
ACACAGGCGCACCGAGAAGATAAGAACCGAAAAGCAGCGAACCAGTAAGCAGCGAACCAGTAAGCAGCGAACC
GAAAAGCAGCGAACCGAAAAGCAGCGAACCGAAAAGCAGCTAA -5′ (SEQ ID NO: 6)

DPA 44

5′-
GTGTCCGCGTGGCTCTTCTATTCTTGGTCATTCGTCGCTTGGCTTTTCGTCGCTTGGTCATTCGTCGCTTGGT
CATTCGTCGCTTGGCTTTTCGTCGCTTGGTCATTCGTCG -3′ (SEQ ID NO: 7)
3′-
ACACAGGCGCACCGAGAAGATAAGAACCAGTAAGCAGCGAACCGAAAAGCAGCGAACCAGTAAGCAGCGAACC
AGTAAGCAGCGAACCGAAAAGCAGCGAACCAGTAAGCAGCTAA -5′ (SEQ ID NO: 8)

Target

5′- ATTTATCTGCTGCATGATCCGATAGTGCGAAT -3′
3′- ATAGACGACGTACTAGGCTATCACGCTTANNNN -5′ (SEQ ID NO: 9)

SLA 13

5′- NATCTAGATGCACTCCCGGACCTC -3′
3′- ATCTACGTGAGGGCCTGGAG -5′
(SEQ ID NO: 10)

SLA 14

5′- NATGTAGATGCACTCCCGGACCTC -3′
3′- ATCTACGTGAGGGCCTGGAG -5′
(SEQ ID NO: 11)

SLA 24

5′- NCGATAGATGCACTCCCGGACCTC -3′
3′- ATCTACGTGAGGGCCTGGAG -5′
(SEQ ID NO: 12)

SLA 44

5′- NGTATAGATGCACTCCCGGACCTC -3′
3′- ATCTACGTGAGGGCCTGGAG -5′
(SEQ ID NO: 13)

BLA 13-1

5′- NBTCATGAGCTGGGCGGCACGTAT -3′
3′- TACTCGACCCGCCGTGCATA -5′
(SEQ ID NO: 14)

BLA 13-2

5′- NAVCATGAGCTGGGCGGCACGTAT -3′
3′- TACTCGACCCGCCGTGCATA -5′
(SEQ ID NO: 15)

BLA 13-3

5′- NATDATGAGCTGGGCGGCACGTAT -3′
3′- TACTCGACCCGCCGTGCATA -5′
(SEQ ID NO: 16)

In Table I, "N" represents any of the bases G, C, T and A; "B" represents any of the bases G, C and T; "D" represents any of the bases A, C and T; "H" represents any of the bases A, C and T; and "V" represents any of the bases A, G and C.

Reactions were carried out where three of the following adapters were ligated together (see Table I):

1) a Design Polymer Adapter (DPA)

2) a target molecule with a 4 nt 5' overhang representing all 256 possible permutations of 4 nucleotides ('NNNN')

3) a Specific Ligation Adapter (SLA) meant to ligate specifically to a subset ($1/64^{th}$) of the target molecules.

Based on its 4 nt overhang, the SLA was supposed to ligate to only 4 out of the 256 permutations of the target.

There were four variants of each DPA (designated 13, 14, 24 and 44) and four of each SLA (designated similarly), see Table I. In some cases, a specific set of three Blocking Adapters (BLA's) were added to each ligation reaction to block all nine single base mismatches possible for a particular reaction (see Table I); there were 3 x 4 variants of each BLA (Table I).

For each reaction, DPA, Target and the corresponding SLA were ligated (at a molecular ratio of DPA:Target:SLA = 64:64:10) in the presence or absence of the specific set of nine BLA's (each BLA was at an equimolar ratio relative to the SLA) using T4 DNA ligase (Fermentas). PCR using the primers described in Table II was performed on $1/20^{th}$ of the ligation reaction to amplify the ligation products.

Table II: PCR and Extension primers (in 5' to 3' orientation)

Forward PCR primer	ACGTTGGATGTGTGTCCGCGTGGCTCTTCT	(SEQ ID NO: 17)
Reverse PCR primer	ACGTTGGATGATGGGCTTTTGAGGTCCGGGAGTG	(SEQ ID NO 18)
Extension primer	GAGGTCCGGGAGTGCATCTA	(SEQ ID NO: 19)

Targets ligated to BLAs could not be amplified during this PCR due to the different sequence at their 3' end (upper strand). PCR products were isolated and concentrated using the MinElute PCR Cleanup Kit (Qiagen).

The cleaned, concentrated PCR products were used in an extension reaction following the Homogenous Mass Extend protocol described in the Mass ARRAY User's Manuals (Sequenom). Basically, the reaction consisted of the product of the PCR amplified ligation product, an extension primer complementary to the sequence 5' of the base to be investigated (see Table II), a 'Stop mix' (a specific mixture of equimolar amounts of one NTP in the dNTP form and the remaining three NTPs in the ddNTPs form), a thermostable

polymerase in 1 x buffer (all components from Sequenom except: ddNTPs from Roche, dNTPs from Amersham). The stop mix was chosen so that a base at the most 3' end of the ligation overhang region resulting from a correct (match) ligation would yield a 2-base extension product whereas a base resulting from a mismatch ligation would yield a 1-base extension product.

Extension reactions and the subsequent washes were performed following the Homogenous Mass Extend method (Sequenom). Extension products were spotted on a SpectroCHIP (Sequenom) and separated according to mass as described in the Mass ARRAY User's Manuals (Sequenom). The results were a set of peaks representing unextended extension primer and (1-base and 2-base) extension products. The intensities of these peaks were transferred manually to a spreadsheet. The relative intensities of the 2-base extension product (representing the match ligation extension product) versus the total intensity of the extension products was calculated.

The results of the experiments are shown in Table III.

Table III: Percentage match ligation extension product of the total extension product for the 3'-most base of the overhang

Reaction (overhang)	Without Blocking Adapters	With Blocking Adapters
13 (NATC)	84.4 %	88.0 %
14 (NATG)	100.0 %	100.0 %
24 (NCGA)	100.0 %	100.0 %
44 (NGTA)	75.8 %	84.4 %

These results indicate that two out of the four ligation reactions (numbers 14 and 24) did not produce a detectable peak for a mismatch ligation product at the 3'-most position. For the other two reactions (13 and 44) the peak intensities for the match-ligation extension product detected represented 84.4% (reaction 13) and 75.8% (reaction 44) of the total extension product intensity, respectively, in the reactions without Blocking Adapters. These intensities were higher when

Blocking Adapters were included in the reactions: 88.0% (reaction 13) and 84.4% (reaction 44). This indicates that blocking adapters helped to reduce mismatch ligations occurring at the 3'-most position.

CLAIMS

1. A method for improving the specificity of a ligation reaction carried out between a first double stranded polynucleotide having a single stranded portion and a second polynucleotide having a complementary single stranded portion, said second polynucleotide being present in a sample comprising a mixture of different polynucleotides, comprising:

contacting the sample, under hybridising conditions, with the first polynucleotide and one or more third polynucleotide(s), wherein the third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base substitution, and carrying out a ligation reaction.

2. A method according to claim 1, wherein the third polynucleotide is a double stranded polynucleotide having said single stranded portion.

3. A method according to claim 1 or claim 2, wherein the single stranded portion on each of the first, second and third polynucleotides is from 3 to 6 bases in length.

4. A method according to claim 3, wherein the single stranded portion is 4 bases in length.

5. A method according to any preceding claim, wherein the single stranded portion of the third polynucleotide differs from the single stranded portion of the first polynucleotide by one base.

6. A method according to any preceding claim, wherein the ligase is T4 DNA ligase.

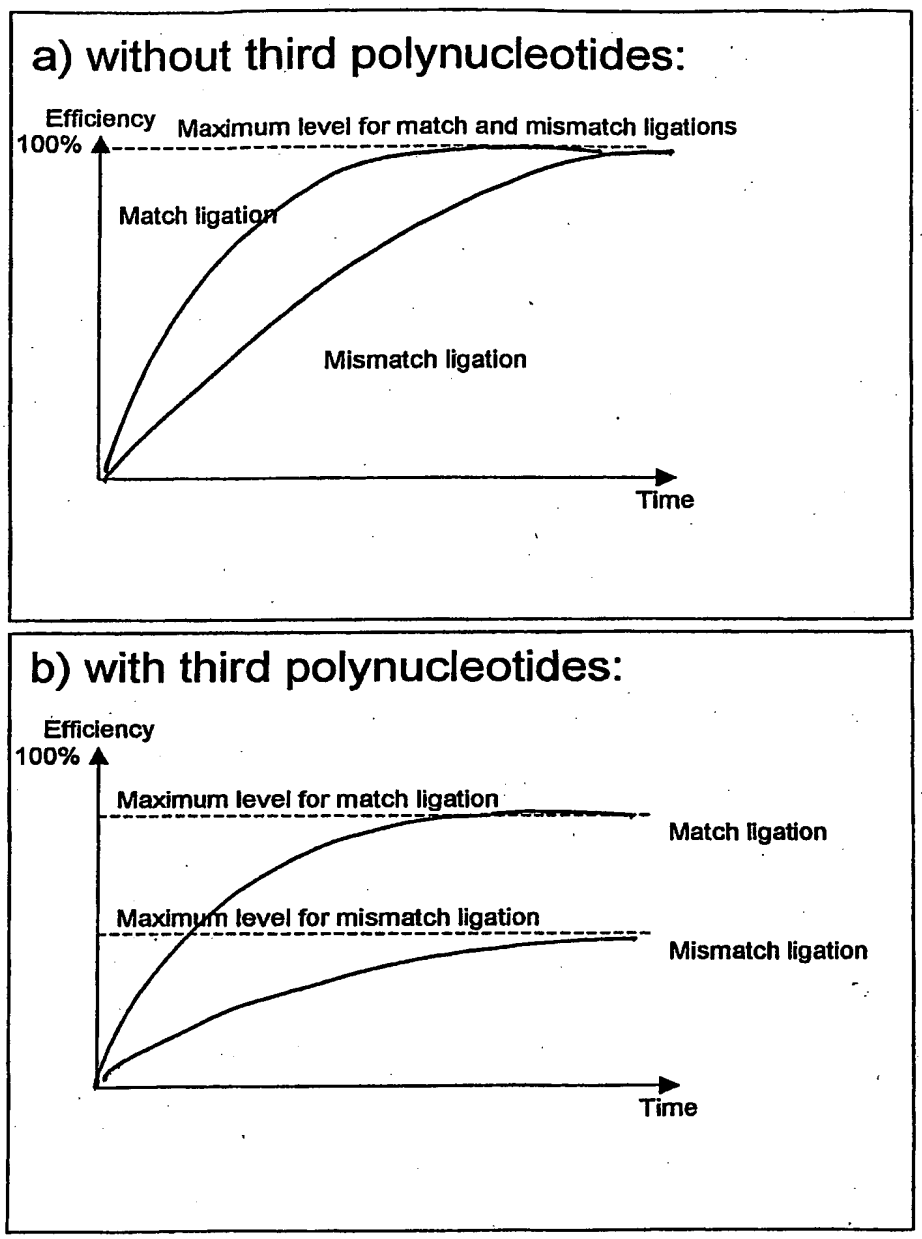
a) without third polynucleotides:
Efficiency
100%
Maximum level for match and mismatch ligations
Match ligation
Mismatch ligation
Time
b) with third polynucleotides:
Efficiency
100%
Maximum level for match ligation
Match ligation
Maximum level for mismatch ligation
Mismatch ligation
Time

Figure 1

INTERNATIONAL SEARCH REPORT

Intern. al Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WU D Y ET AL: "SPECIFICITY OF THE NICK-CLOSING ACTIVITY OF BACTERIOPHAGE T4 DNA LIGASE" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 76, 1989, pages 245-254, XP001061707 ISSN: 0378-1119 the whole document	1-6
Y	WO 03/072812 A (EPIGENOMICS AG; ADORJAN, PETER; BURGER, MATTHIAS; MAIER, SABINE; LESCH) 4 September 2003 (2003-09-04) page 19, paragraph 2	1-6
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 April 2005	17/05/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

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Interna ıl Application No
PCT/GB2005/000225

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Interna il Application No
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2005/000225

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [X] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in computer readable form

   [X] furnished subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference JWJ01013WO	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/GB2005/000225	International filing date *(day/month/year)* 21.01.2005	Priority date *(day/month/year)* 23.01.2004
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant LINGVITAE AS et al.		

1. This report is the International preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☐ *sent to the applicant and to the International Bureau)* a total of sheets, as follows:
      ☐ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in Item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:
   - ☒ Box No. I Basis of the opinion
   - ☐ Box No. II Priority
   - ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - ☐ Box No. IV Lack of unity of invention
   - ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - ☐ Box No. VI Certain documents cited
   - ☐ Box No. VII Certain defects in the international application
   - ☐ Box No. VIII Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23.11.2005	09.12.2005
Name and mailing address of the International preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized Officer Molina Galan, E Telephone No. +31 70 340-3560

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2005/000225

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-10 as originally filed

Sequence listings part of the description, Pages

1-6 as originally filed

Claims, Numbers

1-6 as originally filed

Drawings, Sheets

1 as originally filed

☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-6
	No:	Claims	
Inventive step (IS)	Yes:	Claims	2-6
	No:	Claims	1
Industrial applicability (IA)	Yes:	Claims	1-6
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this report has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☒ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed

   ☐ filed together with the international application in computer readable form

   ☒ furnished subsequently to this Authority for the purposes of search and/or examination

   ☒ received by this Authority as an amendment on

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional observations, if necessary:

Re Item V.

1 Reference is made to the following documents:

D1 : WU D Y ET AL: "SPECIFICITY OF THE NICK-CLOSING ACTIVITY OF BACTERIOPHAGE T4 DNA LIGASE" GENE, ELSEVIER BIOMEDICAL PRESS. vol. 76, 1989, pages 245-254, XP001061707 ISSN: 0378-1119

D2 : WO 03/072812 A (EPIGENOMICS AG; ADORJAN, PETER; BURGER, MATTHIAS; MAIER, SABINE; LESCH) 4 September 2003 (2003-09-04)

D3 : WO 98/24933 A (BOSTON PROBES, INC; DAKO A/S; COULL, JAMES, M; HYLDIG-NIELSEN, JENS, J) 11 June 1998 (1998-06-11)

2 INDEPENDENT CLAIM 1

2.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject matter of claim 1 does not involve an inventive step in the sense of Article 33(3)PCT.

2.1.1 Document D1, which is considered to represent the most relevant state of the art to the subject matter of claim 1, discloses a method to increase the specificity of ligation reactions due to mismatch ligations by increasing the reaction temperature or modifying the usual components of the reaction mixture.

2.1.2 The subject-matter of independent claim 1 differs from the disclosure of D1 in that blocking oligonucleotides are used to sequester possible competing targets to increase the specificity.

2.1.3 The problem to be solved by the present invention may therefore be regarded as the provision of an alternative method for increasing specificity of ligation reactions or, in other words, reducing mismatch ligations.

2.1.4 D1 suggest that the problem of mismatch ligation is solved by increasing salt or spermidine concentrations (cf p. 253, right column, §1), where it is stated that T4

DNA ligase would be able to distinguish substrates with one mismatched base pair. This would not however prevent the skilled person from trying to find alternative ways of solving the mismatch ligation problem.

2.1.5 In trying to find alternative solutions he would consider any document in the field of nucleic acid detection, including D2. This document indicates that blocking oligonucleotides are able to "sequester" a background of sequences similar to the target (cf p19, § 2), i.e., are able to prevent extension of primers hybridised to "mismatched" templates. When considering how the method is performed in the reference cited in D2 (Yu et al), it is however clear that the mechanism is different from the one employed in the solution within claim 1. The blocking oligonucleotides, although designed to hybridise to (expected) background, non target, DNA as in the present application, are not designed to compete with the primers used for the amplification but to bind to a region located between the primers. This would thus not suggest to the person skilled in the art that the inclusion of "third" nucleotides differing from the single stranded part of the adapter by at least one base substitution would allow the reduction of the mismatch ligation.

2.1.6 D3 discloses the use of "blocking" probes complementary to non-target sequences to increase the hybridisation specificity of probes complementary to the target sequence (cf claim 1). A condition is however that either the probe or the "blocking" probe is a PNA. The person skilled would thus not expect that if both probes are DNA sequences the "blocking" effect is achieved.

2.1.7 This means at first sight that the prior art cited (in combination) would not suggest the solution proposed to reduce mismatch ligations. The IPEA is however not convinced that the solution proposed in claim able to solve the problem posed itself. Claim 1 includes the use of single stranded sequences as "blocking" nucleic acids. It is not clear if such a competitive reaction would effectively (in terms of thermodynamic conditions" block background nucleic acid from participating in mismatch ligation. The only example in the application is not using single stranded blocking oligonucleotides but blocking adapters, thus with a double stranded portion at least.

2.1.8 This situation is reflected in the features present in claim 2. In this case the IPEA can believe that an adapter presenting modifications in the single stranded portion would effectively be able to "sequester" potential competing substrate differing from the target by one or few mismatches as also this competing substrate would be ligated to the blocking adapter thus preventing participation again in a competitive reaction with the target.

2.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject matter of claim 1 does not involve an inventive step in the sense of Article 33(3)PCT.

2.3 The features disclosed in claim 2 and all the claims dependent to it can be considered to meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT) for the reasons given above..

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92*bis*.1 and Administrative Instructions, Section 422)

To:

GILL JENNINGS & EVERY LLP
Broadgate House
7 Eldon Street
London EC2M 7LH
ROYAUME-UNI

Date of mailing *(day/month/year)*
02 August 2006 (02.08.2006)

Applicant's or agent's file reference
JWJ01013WO

IMPORTANT NOTIFICATION

International application No.
PCT/GB2005/000225

International filing date *(day/month/year)*
21 January 2005 (21.01.2005)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address
GILL JENNINGS & EVERY
Broadgate House
7 Eldon Street
London EC2M 7LH
United Kingdom

State of Nationality | State of Residence

Telephone No.
+44 20 7377 1377

Facsimile No.
+44 20 7377 1310

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☒ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address
GILL JENNINGS & EVERY LLP
Broadgate House
7 Eldon Street
London EC2M 7LH
United Kingdom

State of Nationality | State of Residence

Telephone No.
+44 20 7377 1377

Facsimile No.
+44 20 7377 1310

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

☐ the receiving Office
☐ the International Searching Authority
☒ the International Preliminary Examining Authority
☐ the designated Offices concerned
☒ the elected Offices concerned
☐ other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 82 70

Authorized officer
Blanc Veronique
Facsimile No. +41 22 338 82 70
Telephone No. +41 22 338 96 66

PATENT COOPERATION TREATY

PCT

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43*bis*.1)

Applicant's or agent's file reference see form PCT/ISA/220	Date of mailing *(day/month/year)* see form PCT/ISA/210 (second sheet) **FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB2005/000225	21.01.2005	23.01.2004

International Patent Classification (IPC) or both national classification and IPC
C12Q1/68

Applicant
LINGVITAE AS

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:

European Patent Office - P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Pays Bas
Tel. +31 70 340 - 2040 Tx: 31 651 epo nl
Fax: +31 70 340 - 3016

Authorized Officer

Molina Galan, E

Telephone No. +31 70 340-3560

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2005/000225

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☒ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed.

   ☐ filed together with the international application in computer readable form.

   ☒ furnished subsequently to this Authority for the purposes of search.

3. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-6
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-6
Industrial applicability (IA)	Yes:	Claims	1-6
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V.

1 Reference is made to the following documents:

D1 : WU D Y ET AL: "SPECIFICITY OF THE NICK-CLOSING ACTIVITY OF BACTERIOPHAGE T4 DNA LIGASE" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 76, 1989, pages 245-254, XP001061707 ISSN: 0378-1119

D2 : WO 03/072812 A (EPIGENOMICS AG; ADORJAN, PETER; BURGER, MATTHIAS; MAIER, SABINE; LESCH) 4 September 2003 (2003-09-04)

D3 : WO 98/24933 A (BOSTON PROBES, INC; DAKO A/S; COULL, JAMES, M; HYLDIG-NIELSEN, JENS, J) 11 June 1998 (1998-06-11)

2 INDEPENDENT CLAIM 1

2.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject matter of claim 1 does not involve an inventive step in the sense of Article 33(3)PCT.

2.1.1 Document D1, which is considered to represent the most relevant state of the art to the subject matter of claim 1, discloses a method to increase the specificity of ligation reactions due to mismatch ligations by increasing the reaction temperature or modifying the usual components of the reaction mixture.

2.1.2 The subject-matter of independent claim 1 differs from the disclosure of D1 in that blocking oligonucleotides are used to sequester possible compeeting targets to increase the specificity.

2.1.3 The problem to be solved by the present invention may therefore be regarded as the provision of an alternative method for increasing specificity of ligation reactions.

2.1.4 In view of D2 the solution proposed in claim 1 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) as it is well known to use competing probes or primers to "sequester" a background of sequences similar to the target (cf p19, § 2 or D3, claims)

2.1.5 Therefore the features disclosed in D1 and D2 would be combined by the skilled person, without exercise of any inventive skills in order to solve the problem posed. The proposed solution in independent claim 1 thus cannot be considered inventive (Article 33(3) PCT).

2.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject matter of claim 1 does not involve an inventive step in the sense of Article 33(3)PCT.

3 DEPENDENT CLAIMS 2-5

Dependent claims 2-5 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).